Insulin-Like Growth Factor-II Regulates the Expression of Vascular Endothelial Growth Factor by the Human Keratinocyte Cell Line HaCaT

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Psoriasis is a chronic, relapsing skin disease characterized by enhanced angiogenesis. The pathogenetic process resulting in hypervascularity remains to be further investigated. It has been reported that a potent angiogenic factor, vascular endothelial growth factor (VEGF) is overexpressed in psoriatic epidermis and that the level of insulin-like growth factor II (IGF-II) is significantly elevated in the tissue fluid and serum of the psoriatic lesion. We considered the possibility that IGF-II might function as a paracrine inducer of VEGF. Here, we demonstrated that exposure of HaCaT keratinocytes to IGF-II induced both mRNA and protein expression of VEGF through the MAP kinase (extracellular signal-regulated kinase (ERK2) pathway. Particularly, we determined that phosphorylation of ERK2 but not p38 and JNK1/2 was activated by IGF-II in a time-dependent manner. Additionally, we found that IGF-II treatment induced the expression of MDM2 through the MAP kinase pathway. Moreover, the increase of MDM2 resulted in decreased levels of p53 followed by increased expression of HIF-1α and VEGF. Taken together, these results suggest that IGF-II enhances the expression of VEGF in HaCaT cells by increasing HIF-1α levels.

Key words: angiogenesis/HaCaT/IGF-II/VEGF
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Psoriasis is a common, chronic skin disease characterized by recurrent erythematous skin plaques that exhibit epidermal hyperplasia (Weinstein *et al*, 1985; Baker and Fry, 1992), a variable inflammatory cell infiltrate, and angioproliferation (Braverman and Keh-Yen, 1986). Capillaries in the papillary dermis of lesions are elongated, dilated, tortuous, and hyperpermeable (Mordovtsev and Albanova, 1989). These findings indicate a critical pathogenetic role for microvascular expansion in lesional skins and have led to the suggestion that psoriasis is an angiogenesis-dependent disease (Folkman, 1972; Beranek, 1990; Creamer *et al*, 1997).

Previous studies demonstrated that a potent angiogenic factor vascular endothelial growth factor (VEGF) is overexpressed in psoriatic epidermis. Two tyrosine kinase receptors for VEGF, Flt-1, and KDR, are also overexpressed in psoriatic dermal microvessels (Detmar *et al*, 1994). These reports suggest that VEGF might play an important role in the pathogenesis of psoriasis. But the mechanisms responsible for overexpression of VEGF in psoriasis are still unclear.

Insulin-like growth factor II (IGF-II) is homologous to proinsulin in both sequence and structure (Cohick and Clemmons, 1993). IGF-I and IGF-II can stimulate mitogenesis in a variety of cells (Daaghaday and Rotwein, 1989; Stewart Claire and Rotwein, 1996), including normal and transformed human keratinocytes (Neely *et al*, 1991). Moreover, the level of IGF-II but not IGF-I was significantly elevated in serum and blister fluid from psoriatic lesions (Xu *et al*, 1996); however, the function of IGF-II in psoriasis has not been well investigated. We have previously demonstrated that IGF-II-induced VEGF expression in human hepatoblastoma HepG2 cells (Kim *et al*, 1998). These findings point to the possibility that IGF-II induces VEGF expression and subsequently activates angiogenesis in psoriasis as well.

It has been reported that IGF-II functions via IGF-I receptors as a potent mitogen in many cell types including keratinocytes (Neely *et al*, 1991; Hodak *et al*, 1996). IGF-I activates extracellular signal-regulated kinase (ERK1) and ERK2 (MAP kinase) in cultured rat cardiac myocytes and HCT116 human colon carcinoma cells through IGFR-I (Foncea *et al*, 1997; Fukuda *et al*, 2002). Therefore, these findings suggested that IGF-II induction of VEGF expression might be mediated through MAP kinase pathway.

HIF-1, a basic–helix–loop–helix–PAS transcription factor, activates transcription of genes whose protein products increase O_2 availability or promote metabolic adaptation to

Abbreviations: ERK, extracellular signal-regulated kinase; HIF-1α, hypoxia-inducible factor-1α; IGF-II, insulin-like growth factor II; VEGF, vascular endothelial growth factor

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low oxygen tension. Examples include erythropoietin, VEGF, glucose transporters, and glycolytic enzymes. Previous studies demonstrated that exposure of human embryonic kidney 293 cells to IGF-II-induced HIF-1α expression (Feldser *et al*, 1999). The precise mechanism by which IGF-II induced HIF-1α expression, however, has not been investigated. HIF-1 consists of two subunits, HIF-1α and HIF-1β (Wang *et al*, 1995). A decrease in cellular O_2 tension leads to elevation of HIF-1 activity via stabilization of the HIF-1α protein; conversely, ubiquitin-mediated proteolysis of HIF-1α on re-exposure to a normoxic environment results in rapid decay of HIF-1 activity (Semenza and Wang, 1992; Wang *et al*, 1995; Salceda and Caro, 1997; Huang *et al*, 1998; Kallio *et al*, 1999). Previous studies demonstrated that genetic inactivation of p53 in cancer cells provides a potent stimulus for tumor angiogenesis and that p53 inhibits HIF-1 activity by targeting the HIF-1α subunit for ubiquitination and proteosomal degradation (Ravi *et al*, 2000). Conversely, the loss of p53 enhances HIF-1-dependent expression of VEGF in tumor cells (Ravi *et al*, 2000).

To investigate whether angiogenic properties of IGF-II are mediated through the induction of VEGF synthesis by the abnormal keratinocytes characteristic of psoriasis, we examined effects of IGF-II on the induction of VEGF in the immortalized HaCaT keratinocyte cells. Our results demonstrated that IGF-II induces VEGF expression by upregulating HIF-1α through the MAP kinase (ERK1/2), MDM2, and p53 pathways.

Results

IGF-II induces the expression of VEGF in HaCaT keratinocytes Since IGF-II but not IGF-I was increased abundantly in psoriatic lesions (Xu *et al*, 1996), we supposed the role of IGF-II in psoriasis to be important. To determine whether IGF-II might be involved in psoriatic angiogenesis, we asked if IGF-II might induce the expression of VEGF in HaCaT keratinocytes. As shown in Fig 1, VEGF mRNA level was approximately 4-fold higher in IGF-II-treated HaCaT cells than that of untreated controls (Fig 1*A*). To confirm our *in vitro* model, we examined VEGF expression in HaCaT under conditions of reduced oxygen (1% O_2), a strong stimulus for VEGF (Detmar *et al*, 1997). As expected, hypoxia strongly induced the expression of VEGF mRNA (Fig 1*A*).

We then performed western blot analysis of VEGF in the conditioned media and protein extracts from HaCaT cells after treatment with IGF-II and found about 2.3-fold increase in secreted VEGF levels (Fig 1*B*). As shown in Fig 1*C*, induction of VEGF protein level was concentration dependent with the maximal effect at 200 ng per mL of IGF-II. Moreover, we performed western blot analysis at several time points (2, 4, 8, and 16 h). Induction of VEGF by IGF-II was detected at 16 h but the level was lower than at 24 h (data not shown). Therefore, we performed the following experiments to delineate the mechanisms involved in expression of VEGF at 24 h. Loading protein levels were equivalent as measured by immunoblot using antibody against α-tubulin. These data demonstrated that IGF-II strongly upregulates VEGF mRNA and protein levels in HaCaT cells.

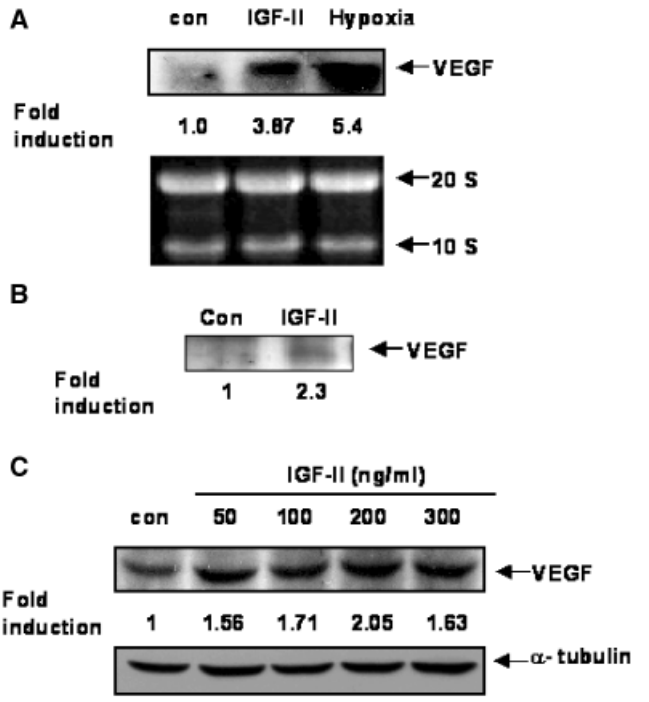

Figure 1
Insulin-like growth factor II (IGF-II) increases the expressions of vascular endothelial growth factor (VEGF) mRNA and protein in HaCaT keratinocytes. (*A*) Northern blot analysis of total RNA (20 μg per lane) was performed. HaCaT cells were incubated with 100 ng per mL IGF-II under 1% hypoxia for 24 h. The lower panels display an image of the respective ethidium bromide (EtBr)-stained RNA gel to demonstrate even loadings. Western blot analysis of the conditioned media (*B*) and total cell lysates (*C*) were performed. HaCaT cells were incubated with 100 ng per mL IGF-II (*B*) and increasing concentrations of IGF-II (50–300 ng per mL) for 24 h (*C*). Con, control. The data are representative of three independent experiments.

IGF-II activates ERK1/2 phosphorylation in HaCaT cells IGF-II functions via IGF-I or IGF-II receptors as a potent mitogen for many cell types (Neely *et al*, 1991) and IGFR-I has been reported to activate ERK1/2 in rat cardiac myocytes and HCT116 human colon carcinoma cells (Foncea *et al*, 1997, 2002). To investigate whether IGF-II would activate ERK1/2 in keratinocytes, we examined phosphorylation status of ERK1/2 in HaCaT cells. Activation of ERK1/2 was determined by western blotting with phospho-specific anti-ERK1/2, which detects only phosphorylated, activated ERK1/2. Selective increases in phospho-ERK2 were seen within 5 min of stimulation, peaked at 30 min (Fig 2*A*) and returned to baseline levels after 2 h (data not shown). In contrast, treatment with IGF-II had no effect on members of the MAP kinase pathway involving p38 (Fig 2*B*) or JNK1/2 (Fig 2*C*).

To confirm the importance of the ERK2 pathways in response to stimulation with IGF-II, we treated cells with PD98059 that specifically inhibits the upstream ERK activator, MAP kinase/ERK kinase (MEK) (Alessi *et al*, 1995). As a control, we also treated the cells with SB203580, a specific inhibitor of p38. As shown in Fig 3*A*, IGF-II-induced phosphorylation of ERK2 was almost completely inhibited by addition of PD98059 at 50 μM. The p38 inhibitor, SB203580 (15 μM) had no effect on ERK2 phosphorylation.

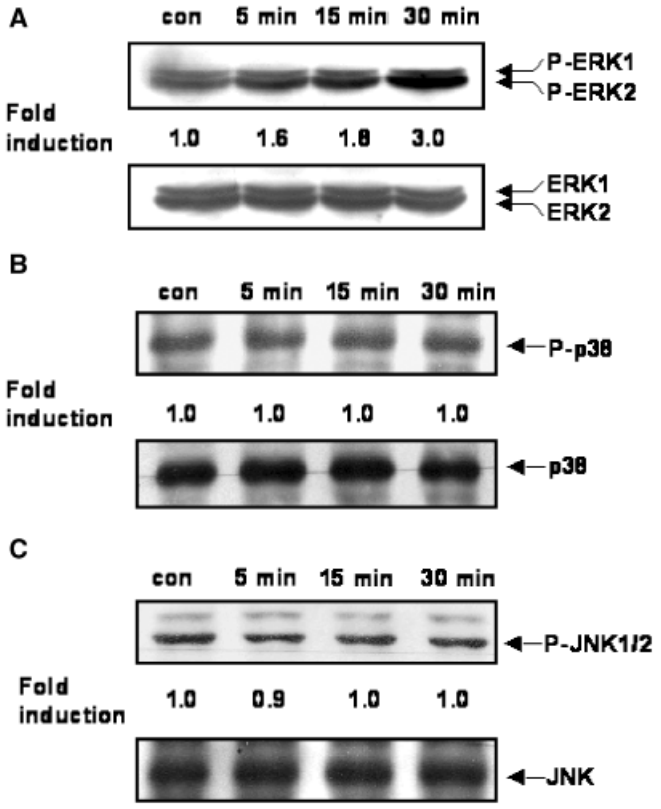

Figure 2
Time-dependent activation of extracellular signal-regulated kinase (ERK1/2) by insulin-like growth factor II (IGF-II). Confluent HaCaT cells were treated with IGF-II (100 ng per mL) and monitored for 5–30 min. Total cell lysates were extracted at the indicated times, and then immunoblotted with anti-phospho ERK1/2, anti-phospho p38, anti-phospho JNK1/2, ERK1/2, p38, or JNK1/2 antibodies as described Materials and Methods. Con, control. The data are representative of three independent experiments.

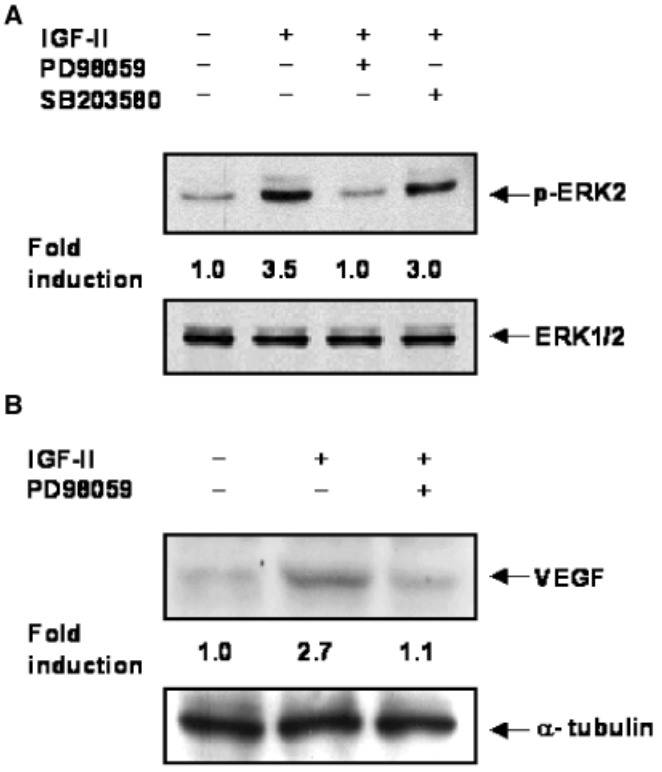

Figure 3
Inhibition of ERK1/2 by PD98059 after IGF-II treatment. HaCaT cells were pretreated for 30 min with PD98059 (50 μM) or SB203580 (15 μM) before treatment of IGF-II (100 ng per mL) and then incubated for 24 h. Cell lysates (40 μg per lane) were isolated and immunoblot analysis was performed with anti-phospho ERK1/2, total ERK1/2 antibodies (*A*) or anti-VEGF antibody (*B*). Relative protein amounts in each sample were checked by expression of α-tubulin. These data are repeated by three times individually.

The concentrations of MAP kinase inhibitors used in these experiments did not affect cell viability. In control experiments, the same amount of DMSO used as a vehicle did not affect cell viability and activation of MAP kinases. The involvement of ERK-signaling pathway on IGF-II-induced VEGF expression was determined by western blot analysis. As shown in Fig 3*B*, treatment with PD98059 attenuated IGF-II-induced VEGF expression in HaCaT keratinocytes. Therefore, these results suggest that VEGF induction by IGF-II may be mediated by the activation of ERK.

Reduction of p53 but induction of MDM2 by IGF-II in HaCaT cells Because psoriasis is a hyperproliferative skin disease and p53 expression is reduced in psoriatic lesions (Michel *et al*, 1996), we studied whether IGF-II was able to reduce the expression of p53 in HaCaT cells. p53 was analyzed by western blot analysis in IGF-II-treated HaCaT cells. In IGF-II-treated cells, p53 levels were reduced at both 16 and 24 h after treatment with IGF-II (100 ng per mL) (Fig 4).

To investigate the mechanism responsible for IGF-II-induced changes in p53 expression, we first examined the expression of MDM2 that targets p53 in the cytoplasm for destruction in proteosome-dependent pathways. As shown in Fig 5*A*, MDM2 levels increased concentration dependently with treatment of IGF-II. In addition, MDM2 was significantly induced at 16 and 24 h in a time-dependent manner (Fig 5*B*). The exposure of HaCaT cells to IGF-II in the presence of PD98059 attenuated MDM2 expression to the basal level (Fig 5*C*). In contrast, the expression of p53 was recovered by co-treatment of PD98059 to the basal

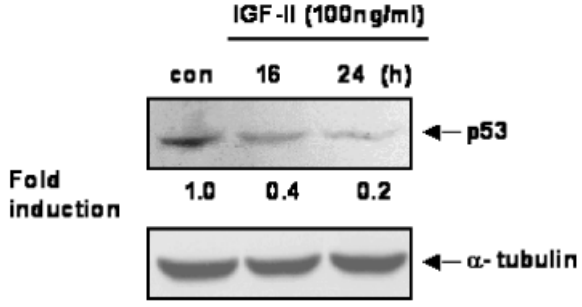

Figure 4
Reduced level of p53 by IGF-II in HaCaT cells. HaCaT cells were treated with IGF-II (100 ng per mL) for 16 or 24 h. Immunoblot analysis of total cell lysates (40 μg per mL) was performed using anti-p53 antibody. Relative protein amounts in each sample were checked by expression of α-tubulin. Con, control. The data are representative of three independent experiments.

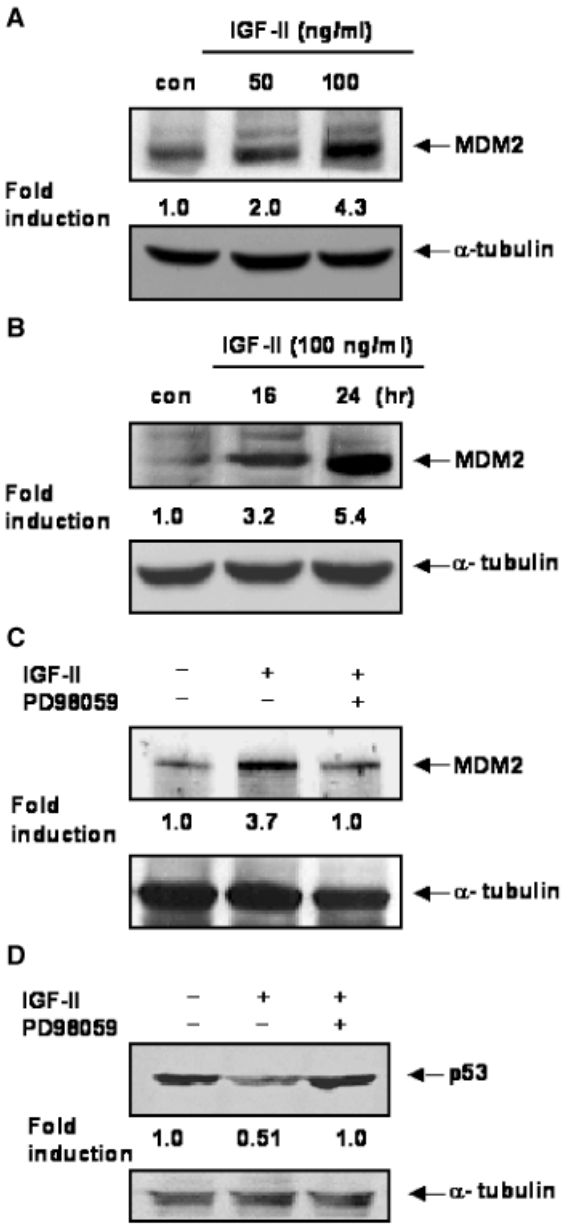

Figure 5
Induced level of MDM2 by IGF-II in a concentration- and a time-dependent manner in HaCaT cells. (*A*) HaCaT cells were treated with IGF-II (50 or 100 ng per mL) for 24 h. Immunoblot analysis of total cell lysates (40 μg per mL) was performed using anti-MDM2 antibody. (*B*) HaCaT cells were treated with 100 ng per ml IGF-II for 16 or 24 h. Following total proteins (40 μg per lane) were isolated and immunoblotting was performed with anti-MDM2 antibody. (*C*, *D*) HaCaT cells were pretreated for 30 min with PD98059 (50 μM) before treatment of IGF-II (100 ng per mL) and incubated for 24 h. Following total proteins (40 μg per lane) were isolated and immunoblotting was performed with anti-MDM2 antibody (*C*) or anti-p53 antibody (*D*). Relative protein amounts in each sample were checked by expression of α-tubulin. Con, control. The data are representative of two experiments.

level (Fig 5*D*), suggesting that IGF-II might downregulate p53 by MDM2 through the ERK signaling pathway.

Induction of HIF-1α by IGF-II in HaCaT cells Recently, it has been reported that p53 reduced the stability and expression level of HIF-1α in cancer cells (Ravi *et al*, 2000)

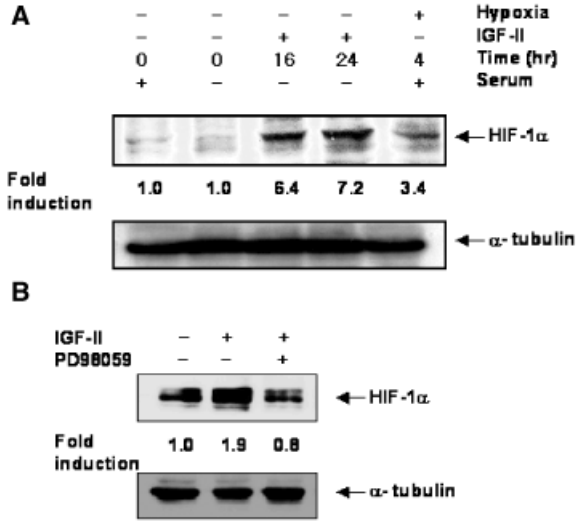

Figure 6
IGF-II induces the protein level of HIF-1α through MAP kinase in HaCaT cells. (*A*) Immunoblot analysis of total lysates (40 μg) was performed using anti-HIF-1α antibody. HaCaT cells were incubated with 100 ng per mL IGF-II for 16 or 24 h or under hypoxia (1% O_2) in the presence of 10% serum for 4 h. (*B*) HaCaT cells were pretreated for 30 min with PD98059 (50 μM) before treatment of IGF-II (100 ng per mL) and incubated for 24 h. Following total proteins (40 μg per lane) were isolated and immunoblot analysis was performed with anti-HIF-1α antibody. Relative protein amounts in each sample were checked by expression of α-tubulin. The data are representative of three experiments.

and IGF-II-induced expression of HIF-1α in human embryonic kidney 293 cells (Feldser *et al*, 1999). In this study, IGF-II was found to reduce the level of p53 mediated by Mdm2 in HaCaT cells, which might increase HIF-1α expression by abrogating p53-mediated HIF-1α degradation. Thus, to determine whether IGF-II induces HIF-1α in keratinocytes, western blot analysis for HIF-1α was performed in HaCaT cells. As shown in Fig 6*A*, IGF-II induced the protein expression of HIF-1α in HaCaT cells. Because hypoxia is a potent inducer of HIF-1α, hypoxia was used as a positive control for HIF-1α (Fig 6*A*). The exposure of HaCaT cells to IGF-II in the presence of PD98059 downregulated HIF-1α to the basal level (Fig 6*B*). Therefore, IGF-II induces the expression of HIF-1α, which in turn might upregulate the expression of VEGF.

Discussion

In several conditions including wound healing, bullous diseases with subepidermal blister formation, epidermal keratinocytes overexpress VEGF mRNA, and dermal microvascular endothelial cells overexpress mRNA encoding both VEGF receptors, Flt-1 and KDR (Brown *et al*, 1992). VEGF and its receptors are also overexpressed in many tumors such as squamous cell carcinomas of the skin (Weninger *et al*, 1996) and in chronic inflammatory states including psoriasis (Detmar *et al*, 1994). All of these conditions are characterized by microvascular hyperpermeability and angiogenesis (Dvorak *et al*, 1986). It has been reported that VEGF is highly induced in many tumors by a

number of diverse stimuli, including nitric oxide, glucose, hormones, cytokines, angiogenic factors themselves, and hypoxia (Brown *et al*, 1997), but in psoriasis, regulators involved in VEGF induction until now remain unknown.

Recently it has been reported that HaCaT cells synthesize IGF-II, IGFBP-3, IGFBP-6, IGFBP protease but not IGF-I. The presence of these components of the IGF system indicates that an autocrine of IGF-II loop may be a potent contribution to psoriatic keratinocyte proliferation. The IGF-I level in tissue fluid from the involved psoriatic area was similar to that from the uninvolved areas but the IGF-II level was significantly elevated in the involved areas (Xu *et al*, 1996). These findings indicated that IGF-II has more important roles than IGF-I in psoriasis. IGF-II is a well-known mitogen for keratinocytes and also has a potent angiogenic activity (Daaghaday and Rotwein, 1989; Lee *et al*, 2000). It is still questionable, however, whether IGF-II is responsible for angiogenesis seen in psoriasis. Previously, we demonstrated that IGF-II could induce VEGF expression in human hepatoblastoma cell line HepG2 cells (Kim *et al*, 1998). Therefore, we proposed that IGF-II might induce the expression of VEGF and play important roles in inducing angiogenesis in psoriasis.

In this study, we demonstrated that IGF-II functioned as a potent inducer of VEGF synthesis by the immortalized keratinocyte cell line HaCaT (Fig 1). Although the correlation between HaCaT cells and psoriasis is unclear, it is still possible that IGF-II has a similar effect on the induction of VEGF in the psoriatic diseases. Moreover, in many cases, VEGF induced by various factors has a potent angiogenic effect; therefore, IGF-II and IGF-II-induced VEGF may both contribute to psoriatic angiogenesis.

The significance of IGF-II-induced VEGF was underlined by the fact that VEGF was not induced by other cytokines associated with psoriasis, such as interleukin-1, interleukin-6, interleukin-8, tumor necrosis factor-α, and TGF-β in human keratinocytes (Detmar *et al*, 1995; Trompezinski *et al*, 2002). Recently, it was reported that hepatocyte growth factor/scatter factor (HGF/SF)-induced VEGF expression in human keratinocytes (Gille *et al*, 1998; Wojta *et al*, 1999; Reisinger *et al*, 2003), but the mechanism of HGF-induced VEGF expression has not been well defined.

It was reported that antisense oligonucleotides of IGFR-I caused a normalization of the hyperplastic epidermis in psoriasis (Christopher *et al*, 2000) and IGF-II functioned via IGF-I receptors as a potent mitogen in keratinocytes (Neely *et al*, 1991). Thus, the elevated IGF-II level in psoriasis may play an important role in epidermal hyperproliferation through IGFR-I. This suggestion correlates well with the findings that IGFR-I is overexpressed in psoriatic lesion and involved in hyperproliferation of psoriatic epidermis (Krane *et al*, 1992). In addition, IGFR-I is known to transduce extracelluar signals through MAP kinase pathways (Foncea *et al*, 1997, 2002). Therefore, we examined the MAP kinase pathway to investigate signal transduction mechanisms involved in IGF-II-induced VEGF expression. As shown in Fig 2, we demonstrated that ERK2 was phosphorylated by IGF-II in HaCaT keratinocytes. We also found that VEGF expression induced by IGF-II was remarkably inhibited in the presence of a specific inhibitor of the upstream ERK activator MAP kinase/ERK kinase (MEK), PD98059 (Fig 3).

Three structurally related but biochemically and functionally distinct MAP kinase signal transduction pathways include the ERK, c-jun N-terminal kinases (JNK) or stress-activated protein kinase and p38 MAP kinase (Su and Karin, 1996). Individual MAP kinase subtypes appear to have equivalent but distinct upstream kinases that are activated by a wide variety of extracellular stimuli. Some extracellular stimuli appear to activate multiple MAP kinase pathways, whereas other signals activate only a single pathway (Assefa *et al*, 1997). In this study, we found that IGF-II treatment of HaCaT keratinocytes may activate a single MAP kinase pathway, ERK but not p38 or JNK (Fig 2). Because ERK1/2 activation is associated with expression of several genes including MMP-1 (Watts *et al*, 1998), interleukin-6 (Hayashi *et al*, 2000), cyclooxygenase-2 (Niiro *et al*, 1998), and MDM2 (Ries *et al*, 2000), IGF-II may activate many other genes in psoriasis and other pathological conditions associated with elevated expression of IGF-II. Elucidation of other genes induced by IGF-II through ERK1/2 may help to explain the pathogenesis of psoriasis.

The normal p53 tumor suppressor protein mediates both growth arrest and apoptosis whereas mutant forms are associated with tumorigenesis. Interestingly, it was reported that p53 expression was reduced in psoriatic lesions (Michel *et al*, 1996); however, the mechanisms responsible for and consequences of reduced p53 expression in psoriasis had not been investigated. In this report, we found that IGF-II-induced MDM2, a potent inhibitor of p53, might degrade p53 through a proteasome-dependent pathway (Haupt *et al*, 1997; Kubbutat *et al*, 1997) (Figs 4 and 5). It has been reported that activation of the Ras/Raf/MEK/MAP kinase signaling cascade results in elevated levels of MDM2 (Persons *et al*, 2000). We demonstrated that IGF-II induced the activation of MEK and ERK2 in HaCaT cells (Figs 3 and 4) and IGF-II-induced MDM2 was dramatically reduced by PD98059 treatment (Fig 5*C*). Therefore, IGF-II stimulates the expression of MDM2 protein through the Ras/Raf/MEK/MAP kinase cascade.

Recently, it has been reported that p53 repressed HIF-1-stimulated transcription (Blagosklonny *et al*, 1998) through degradation of HIF-1α. Loss of p53 in tumor cells enhances HIF-1α levels and increases the transcriptional activity of VEGF gene under hypoxia (Ravi *et al*, 2000). These results support that amplification of normal HIF-1-dependent responses to hypoxia via loss of p53 function contributes to the angiogenic switch during tumorigenesis.

Therefore, a loss of p53 function by IGF-II-induced MDM2 or p53 mutation given to HaCaT cells might contribute for the induction of HIF-1α. In this report, we showed that the reduced level of p53 and the overexpression of HIF-1α result secondarily in stimulation of VEGF expression in HaCaT cells (Figs 5 and 6).

Moreover, it has been demonstrated that IGF-I can induce the HIF-1 protein expression through the MAP kinase pathway (Fukuda *et al*, 2002). They reported that IGF-I-activated MAP kinase via IGF-1R and ERK activation of the transcriptional factor eIF-4E, and finally, eIF-4E induces synthesis of HIF-1α. Many of these data are consistent with our results. Therefore, we suggest that IGF-II might be involved in the synthesis of HIF-1α induced by the MAP kinase pathway.

Although HaCaT cells are widely used as a model for primary normal keratinocytes, retinoids, or UV irradiation showed different effects on VEGF expression in primary keratinocytes and HaCaT cells (Weninger *et al*, 1998; Mildner *et al*, 1999). Thus we have tried to elucidate IGF-II-mediated HIF-1α/VEGF signaling pathways in primary normal keratinocytes as well. We found, however, that normal primary keratinocytes exerted no significant changes of VEGF, p53, and HIF-1α expressions in response to IGF-II (data not shown), in contrast with HaCaT cells. From these results, we assumed that these different effects between two type cells might come from different culture conditions or unidentified reasons. Therefore, the elucidation of pathogenesis of psoriasis should be dealt with careful examinations, because the correlation between HaCaT cells and primary/psoriatic keratinocytes remains still unclear.

In conclusion, we suggest that IGF-II induces angiogenesis and microvascular permeability by the increased expression of VEGF in HaCaT keratinocytes. The mechanism of VEGF expression might be through a pathway in which IGF-II activates HIF-1α via ERK pathway. The activated ERK induces MDM2, MDM2 in turn degrades p53, and finally the loss of p53 induces HIF-1α in HaCaT cells. These results may provide an important clue to understand possible pathogenetic mechanisms associated with VEGF expression and the function of IGF-II in psoriasis.

Materials and Methods

Recombinant IGF-II was purchased from R&D (Minneapolis, Minnesota). Anti-VEGF antibodies were obtained from Santa Cruz (Santa Cruz, California) and anti-HIF-1α antibody was from BD Transduction Laboratories (BD Biosciences Pharmingen, Chicago, Illinois). Anti-ERK, phospho-ERK, p38, phospho-p38, JNK, phospho-JNK antibodies were kindly provided by Dr Rony Seger (Weizmann Institute of Science, Rehovot, Israel). A specific inhibitor of MEK1, PD98059 and specific inhibitor of p38, SB203580 were obtained from Calbiochem, Inc., La Jolla, California, and stock solutions prepared in dimethyl sulfoxide (DMSO).

Cell culture The differentiated human keratinocyte cell line HaCaT was kindly provided by Dr N. Fusenig, German Cancer Research Center (Heidelberg, Germany). The cells were maintained as monolayer cultures in Dulbecco's modified eagle medium (DMEM; Life Technologies, Gaithersburg, Maryland), supplemented with 10% fetal bovine serum (FBS; Life Technologies), 100 U per mL of penicillin and 100 μg per mL of streptomycin (Life Technologies) at 37°C in a humidified incubator under 5% CO_2. Cells were seeded at a density of 1×10^6 cells/100 mm dishes. After 48 h, cells were washed with serum-free media, replaced with 10 ml of media without FBS and incubated in a humidified incubator (Forma Scientific Inc., Marietta, Ohio) with an interior temperature of 37°C for at least 18 h prior to experiments examining IGF-II treatment.

Northern blot analysis Total RNA were isolated from cells treated with IGF-II (100 ng per mL) (R&D) and hypoxia using TRI reagent (Life Technologies). RNA (20 μg) was electrophoresed on 1.2% agarose/6.6% formaldehyde gels, transferred to Zeta-Probe nylon membrane (Bio-Rad Lab., Richmond, California), and was covalently linked by UV cross-linker (UV Stratalinker 1800; Stratagene, La Jolla, California). Hybridization was then performed with the addition of 20 ng of α-^{32}P-labeled VEGF cDNA probes at 42°C for 16 h. Membrane was washed twice with washing solution (0.5 × SSC/0.1% SDS) at 50°C for 30 min, and exposed to X-ray film with intensifying screens at −70°C for 24 h. α-^{32}P-labeled DNA probes were prepared by using random primer oligonucleotides (Rediprime DNA Labeling System; Amersham Pharmacia Biotech, Buckinghamshire, UK).

Immunoblot analysis Cells were treated with IGF-II for 24 h and lysed with buffer containing 40 mM Tris-HCl, pH 7.4, 10 mM EDTA, 120 mM NaCl, 1 mM dithiothreitol, 0.1% noniodet P-40, and protease inhibitors. Total proteins were electrophoresed using 15% reducing polyacrylamide gels. Immunoblot analysis was performed using anti-VEGF (Santa Cruz), DO1 (anti-p53 antibody, Santa Cruz), MDM2 (Santa Cruz), and HIF-1α (BD Biosciences Pharmingen) polyclonal antibody with emission chemiluminescence-based detection.

Detection of ERK1/2 for analysis of MAPK HaCaT cells were treated with IGF-II (100 ng per mL) for indicated times and immediately lysed in lysis buffer (25 mM Hepes, pH 7.6, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 0.5 mM DTT, 0.1 mM sodium orthovanadate, 20 mM β-glycerophosphate, 4 mM *p*-nitrophenyl phosphate, 20 μg per mL leupeptin, 1 mM PMSF). Total proteins were used for immunoblot analysis with anti-phospho-ERK, phospho-p38, phospho-JNK antibodies, and anti-ERK, anti-p38, anti-JNK antibodies.

Detection of VEGF expression by IGF-II in the conditioned media HaCaT cells were treated with IGF-II (100 ng per mL) for 24 h in serum-free media and immediately the conditioned media was prepared with trichloroacetic acid (TCA) protein precipitation. BCA protein assay was performed to measure the protein concentration. Total proteins were used for immunoblot analysis with anti-VEGF (Santa Cruz) antibody.

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